

Yeast Metallothionein and Applications in Biotechnology

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INTRODUCTION AND HISTORICAL BACKGROUND

The metal-regulated genetic system in yeasts offers a powerful new tool which has not been exploited in the past. Metal-regulated DNA sequences are the most efficient elements for heterologous expression discovered to date. The information on yeast metallothionein (MT) brought together in this review should be useful to the geneticist, the biochemist, and the pharmacologist as well as to the inorganic chemist.

Living organisms have evolved several mechanisms to respond to toxic effects of heavy metals. One of the most common mechanisms is the induction of MTs after uptake of the metal into the cell. MTs are broadly defined as a class of low-molecular-weight cysteine-rich proteins which bind heavy metals (42). MTs are widely distributed throughout living organisms and are fairly well conserved in humans, mammals, plants, and fungi (23, 42). A unique property of this class of proteins is their inducibility in response to the treatment of cells with appropriate metals. For example, resistance to the toxic effects of copper ions in the yeast *Saccharomyces cerevisiae* is mediated by induction of a 6573-dalton cysteine-rich protein, copper-MT (Cu-MT) (9, 21).

In the past few years, with the advent of recombinant deoxyribonucleic acid (DNA) technology, the field of yeast genetics has taken a new turn. Availability of a variety of mutants, defined and mapped by genetic techniques, coupled

with the ability to transform yeasts with cloned genes has enhanced the power of yeast genetics. The marriage between classical yeast genetics and recombinant DNA technology has made *S. cerevisiae* a model system for eucaryotic studies (86).

Although the molecular basis of copper resistance in fungi has only recently been elucidated by cloning the *CUP1* locus, which contains the Cu-MT gene (8, 20, 46), the resistance of yeasts to copper ions was first described in 1951 by Ashida and co-workers (61). They demonstrated that, while laboratory yeast strains rarely grow on agar containing copper, prolonged exposure sometimes leads to the proliferation of dense brown colonies. The color is presumably due to the internalization of copper. From their original studies, they inferred that development of resistance to copper is a two-step process consisting of, first, a slow adaptation on media containing copper and, second, a rapid proliferation of selected colonies. This interpretation is consistent with our current understanding of copper resistance via gene amplification (20). The first genetic definition of copper sensitivity (*cup1⁻*) and copper resistance (*CUP1⁺*) was obtained by Lindegren and co-workers (4), who performed genetic crosses between *CUP1⁺* and *cup1⁻* strains that demonstrate that this trait is mediated by a single gene.

MTs were first purified from horse kidney in 1957 by Margoshes and Vallee (59), who were interested in the biological role of cadmium and zinc. In 1966, Pulido et al. (72) isolated MT from human liver. Since then, MTs have been isolated from a large number of species. The first report on a copper-binding protein from yeast cells was published

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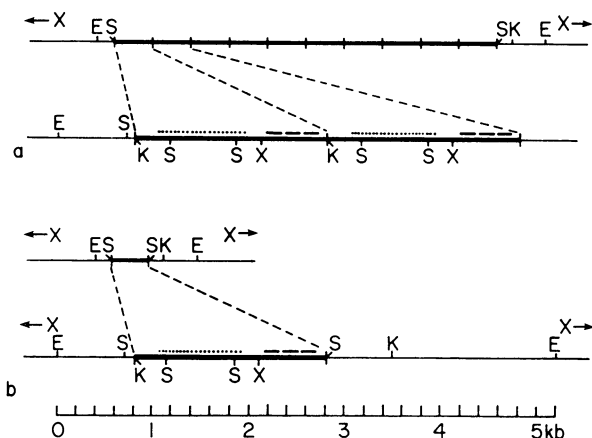


FIG. 1. Restriction maps of (a) $CUP1^+$ and (b) $cup1^-$ strains. (a) The thick solid line segment represents the $CUP1^+$ locus as 10 tandemly iterated 2.0-kb units, each of which is delimited by the vertical lines. The thin line segments represent the external flanking DNA sequences. Shown also are two expanded repeat units. *Sau3A-Sau3A* intervals = 0.7 and 1.3 kb, *Kpn-Kpn* = 2.0 kb, and *Xba-Xba* = 2.0 kb. The thick dashed line denotes the coding region of the MT gene. The dotted line denotes the coding region of an unknown gene. E, *EcoRI*; K, *KpnI*; X, *XbaI*; S, *Sau3A*. (Adapted from reference 21.) (b) The $cup1^-$ locus is composed of only a single unit. The unit and flanking DNA regions are expanded.

in 1975 (70, 71), and since 1975 several laboratories have purified the copper-inducible protein from yeasts and other fungi (52, 56, 63, 65, 66, 70, 71, 83). As a consequence of the different protocols used by individual investigators to purify the copper-inducible yeast protein, variations in molecular weights or biochemical properties have been documented (65, 70, 71). Also, the same protein from *S. cerevisiae* has been referred to in the literature by different names, such as copper-thionein (71), copper-chelatin (70), or Cu-MT (8, 9). However, from a comparison of the protein sequence data from mammalian MT genes (47, 81) and the yeast copper-inducible protein gene (8, 46), it is apparent that the low-molecular-weight cysteine-rich protein belongs to the class of metal-binding proteins designated MT in higher eucaryotes (42). Since the yeast protein is inducible only by copper and not by cadmium or zinc, we refer to it as Cu-MT or yeast MT.

S. cerevisiae contains a single Cu-MT gene, present in the $CUP1$ locus (46), whereas the human MTs are encoded by a multigene family (47). In addition to regulation of expression by cadmium and zinc, the mammalian MT genes are also regulated by glucocorticoids and various other ligands (15, 44, 45). Regulation of MTs by simple ligands such as cadmium, copper, and zinc has made them a useful model in which to study the mechanism of gene regulation.

The present review emphasizes the structure and function as well as regulated gene expression of fungal MTs. Whenever necessary, we have compared fungal and mammalian MTs. In most cases, the word "yeast" refers to strains of *S. cerevisiae*. No comprehensive review on fungal MTs is currently available, but the reader is referred to recently edited volumes which deal with metals and MT (23, 42). A review which primarily deals with mammalian MTs is also available (32). In this review we first describe the structure of the yeast $CUP1$ locus, mechanisms of gene amplification, and copper resistance. The next section catalogs our present knowledge of the known fungal metal-binding or metal-

inducible proteins. Considerable discussion is devoted to regulatory signals of the Cu-MT gene and their role in gene expression. Finally, we describe the commercial applications of the yeast Cu-MT gene and the $CUP1$ locus in biotechnology.

$CUP1$ LOCUS OF *S. CEREVISIAE*

Structure of the $CUP1$ Locus

In genetic terms the copper resistance in *S. cerevisiae* is ascribed to the function of the $CUP1$ locus (4, 20, 92), which is located on chromosome VIII, 42 centimorgans distal to the centromere (20). Copper-sensitive ($cup1^-$) *S. cerevisiae* cells do not grow on media containing >0.15 mM copper. Copper-resistant *S. cerevisiae* ($CUP1^+$) cells contain 10 or more copies of the $CUP1$ locus and can grow on media containing 2 mM copper. The multiple copies of the $CUP1$ locus in $CUP1^+$ strains are tandemly repeated (20). In this respect, the organization of the $CUP1$ locus is similar to that of ribosomal genes in yeasts or other eucaryotes in which the repeating units are tandemly arranged. In general, the *S. cerevisiae* strains are resistant to 1 mM copper and, in most cases, this is directly related to the copies of the $CUP1$ locus present on the chromosomes (92).

The $CUP1$ locus was first cloned by Fogel and Welch (20), who transformed a $cup1^-$ strain with a DNA library and isolated the DNA fragment which confers copper resistance to otherwise copper-sensitive strains. The DNA fragments of the $CUP1$ locus which confer copper resistance in yeasts and encode yeast MT have subsequently been cloned by others (8, 35). Evidence that the $CUP1$ locus is repeated several times in $CUP1^+$ strains was obtained after restriction enzyme analysis of the cloned DNA. Figure 1a shows a genomic restriction map of the tandemly repeated yeast $CUP1$ locus. The *EcoRI* DNA fragment shown contains 10 copies of the $CUP1$ locus. The basic repeating unit " $CUP1$ locus" is composed of 2.00-kilobase (kb) DNA fragments which contain a unique *XbaI* site, two sites for *KpnI*, and *Sau3A* restriction enzymes. The complete nucleotide sequence of the $CUP1$ locus has been determined (46). It appears that the basic repeat unit codes for two genes, one of which codes for a 246-amino acid protein denoted protein X (46). No function has been ascribed to protein X as yet. The smaller messenger ribonucleic acid (RNA) transcribed by the $CUP1$ locus encodes for a 61-amino acid cysteine-rich protein, the yeast MT. Of the two genes present on the $CUP1$ locus, only the MT gene is transcriptionally induced after addition of copper to the cells. Digestion of genomic DNA from a $cup1^-$ strain did not reveal a 2.0-kb DNA fragment, indicating that the $cup1^-$ strain contained a single $CUP1$ locus. This has been confirmed by subsequent cloning of the 5.2-kb *EcoRI* fragment from the $cup1^-$ strain which harbors one copy of $CUP1$ (Fig. 1b) flanked by *KpnI* sites. Deletion of the $CUP1$ locus in *S. cerevisiae* strains which contains a single $CUP1$ has been extremely useful for elucidation of the mechanism of yeast MT gene regulation (see below).

The number of $CUP1$ copies present on chromosomes varies in laboratory yeast strains. Since the repeat unit does not contain any *EcoRI* sites, digestion of the genomic DNA with the enzyme and analysis of the size of *EcoRI*-generated $CUP1$ fragments delineate the number of $CUP1$ copies on the chromosome (21). In a series of studies, Fogel et al. (22) determined that $CUP1^+$ strains carry 2 to 14 copies of the $CUP1$ locus. Continuous growth of laboratory strains in

minimal medium containing >1 mM copper enhances the copper tolerance of yeasts. Selection techniques designed to isolate hyperresistant *CUP1*^r strains appear disomic for chromosome VIII, the chromosome containing the *CUP1* locus (22). The disomic chromosomes exhibit differential *CUP1* gene amplification patterns. Eleven and 14 tandemly organized repeat units are found in chromosome VIII homologues, indicating that mechanisms of copper resistance involve not only amplification of the *CUP1* locus on the chromosomes, but also disomy or aneuploidy of chromosomes VIII.

It is remarkable that all laboratory yeast strains examined thus far contain the 2.00-kb repeat unit of the *CUP1* locus and identical junction sequences between the loci. While most of the laboratory yeast strains have conserved the structure of the *CUP1* locus, industrial yeast strains display considerable heterogeneity in the size of the *CUP1* locus. Three of the wine-making yeast strains were found to display a *CUP1* repeat unit of 1.5 kb, while in another strain (Montrachet), the *CUP1* locus contains repeats of both a 1.7- and a 1.5-kb fragment. Chromosome analysis of this strain reveals that one of the tetrads contains 11 copies of the 1.5-kb *CUP1* repeat while another contains 2 copies of the 1.7-kb *CUP1* repeat (22). Some of the industrial yeast strains display considerable resistance to copper; however, hybridization analysis with the *CUP1* DNA does not reveal any *CUP1* sequences in these strains (22). It is apparent that, in these resistant strains, a mechanism other than amplification of the *CUP1* locus or induction of MT is involved in protection against exogenous copper.

MT Genes from Other Fungi

Progress continues to be made on analysis of metal-regulated genes from other fungi, although the published data are sparse. A copper-inducible cysteine-rich protein has been described in the ascomycete *Neurospora crassa* (56). Recently, the gene for *N. crassa* MT protein has been cloned and the nucleotide sequence has been determined (62). The gene codes for a 26-amino acid protein. The *N. crassa* MT is the smallest MT discovered to date. Another unique property of the *N. crassa* gene is that it is the only fungal MT gene interrupted by an intron (62). Although the protein bears some homology to the mammalian protein, especially at the amino terminus, there is no DNA sequence homology to the mammalian MT genes. The metal-regulatory mechanisms of *S. cerevisiae* and *N. crassa* MTs are presumably similar, as both genes are regulated by copper ions, but no DNA sequence homology is observed in upstream regulatory sequences of the gene. It appears that the two genes may have been evolved through convergent evolution (see subsection, "Evolution of MTs").

When *Schizosaccharomyces pombe*, a fission yeast, is analyzed for MT induction after the cells are challenged with copper, no detectable low-molecular-weight cysteine-rich protein is observed (unpublished data). Similarly, hybridization analysis with the yeast MT gene (*Xba*I-*Kpn*I fragment [9]) with *Schizosaccharomyces pombe* DNA does not show any detectable hybridization under stringent conditions (unpublished results). These data do not exclude the possibility that *Schizosaccharomyces pombe* may contain a metal-binding protein inducible by other metals.

Induction of two copper-binding proteins has been described in the fungus *Dactylium dendroides* (83). One of the proteins is rich in cysteine, while the other copper-binding peptide does not contain any cysteine. It is not known

whether any of these peptides are coded by a gene. (There are examples of metal-binding peptides which are chemically synthesized by plants and do not represent a gene product [see below].)

Human pathogenic fungi. Several fungi pathogenic in humans have been screened for the presence of DNA sequences homologous to the *S. cerevisiae* MT gene. Southern blot and restriction enzyme analysis of the DNA in pathogenic fungi show that one of the *Candida albicans* strains contains DNA sequences which hybridize with *S. cerevisiae* MT (10). In addition, analysis of copper-inducible proteins from this pathogenic strain show similarity to the *S. cerevisiae* MT (10). Detailed restriction enzyme analysis data on the MT locus of *C. albicans* are not available. A nonpathogenic strain of *Candida glabrata* (strain 62) also produces a copper-inducible 4,500-dalton protein, but the *S. cerevisiae* MT probe does not hybridize with *C. glabrata* 62 DNA. DNA sequences homologous to *S. cerevisiae* are present in only a few other yeast strains, and yet copper-inducible protein, which is different in molecular weight from the *S. cerevisiae* MT, is detected in other pathogenic fungi. A comprehensive analysis of protein and the MT gene is required in fungal pathogens of humans before we can understand the evolution of metal resistance in pathogenic fungi in general or evolution of the MT structural gene in particular. Since most of the pathogenic fungi are diploid, they do not lend themselves to conventional genetic studies which are possible with the haploid *S. cerevisiae*. Cloning of a copper-binding protein gene, which could act as a dominant selectable marker, will be an advance for research on pathogenic fungi.

Cadmium-resistant fungi. To date, there is no report of a cadmium-inducible protein in *S. cerevisiae*, but there are some suggestions that cadmium resistance in *S. cerevisiae* is conferred by a low-molecular-weight cadmium-binding protein (41). Cadmium-binding peptides of molecular weights 4,000 and 1,800 have been isolated from the fission yeast *Schizosaccharomyces pombe* (63, 64). The structure of one of the peptides has been determined (64). It is not known whether induction of these cysteine-containing peptides is mediated at the transcriptional level or whether the peptide is coded by a gene.

Mechanisms of *CUP1* Gene Amplification

Copper resistance in *S. cerevisiae* and drug resistance in tissue culture cells, animals, and humans in clinical settings have been ascribed to gene amplification (79, 80). Well-documented reports describe amplification of the dihydrofolate reductase gene in response to the antifolate drug methotrexate (79, 80). Other examples of mammalian gene amplification include increased resistance to *N*-(phosphonacetyl)-L-aspartate mediated by amplification of the CAD locus (85). Increased resistance to cadmium in mouse and Chinese hamster ovary cells is attributed to amplification of MT-I and MT-II genes (13).

Amplification of a particular gene is one of the few mechanisms to achieve increased production of certain macromolecules in the cells. Gene amplification can occur under selection pressure or drug treatment or during development and differentiation. A well-known example of developmental amplification is the repeated ribosomal RNA genes of amphibians and other species which are produced during oogenesis and thereby meet the egg's high requirement for ribosomal RNA (6). Amplification of chorion genes during the development of the late stages of *Drosophila melanogaster*

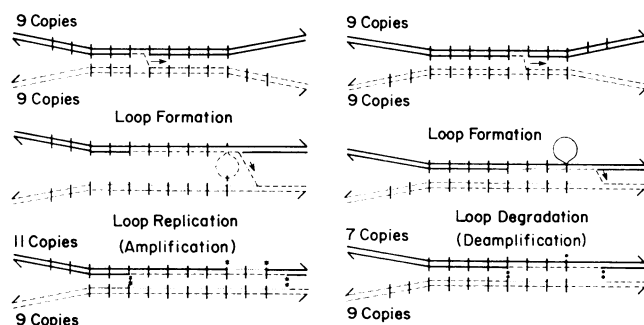


FIG. 2. Schematic model describing amplification and deamplification of the *CUP1* locus. The model proposes a symmetrical strand transfer with unpaired single-stranded loop formation. The double helices of the two different chromatids are shown as double lines (broken and solid). Loop degradation leads to copy number decrease, and loop replication increases copy number (see text).

ter is another well-known example of amplification during differentiation (84).

In mammalian systems, unequal crossing over of sister chromatids and disproportionate replication have been proposed as alternate mechanisms of gene amplification. While the *CUP1* locus of yeasts provides an excellent system to test hypotheses about mechanisms of gene amplification, it is surprising to note that not very many investigators are working on the topic. Laboratory yeast strains which carry single or multiple copies of the *CUP1* locus have been defined. Diploid hybrids can be constructed which are homozygous for copy number of *CUP1* but heterozygous for several independently segregating markers. Cultures from these strains are sporulated, and tetrad analysis is performed to determine the change in the *CUP1* locus by studying the segregation pattern relative to the phenotypic markers. The advantage of this system is that the repeat size of the *CUP1* locus can be directly analyzed by Southern blot analysis. The system has allowed Fogel et al. (22) to distinguish between simple single gene conversion events or multiple conversional events and unequal crossover as mechanisms of *CUP1* gene amplification. They have proposed a model for *CUP1* gene amplification which is shown in Fig. 2 and discussed below.

For detailed discussion of eucaryotic gene amplification mechanisms, the reader is referred to reviews by Stark and Wahl (85) or Schimkie (80). For a general reader who is not familiar with yeast genetics, in simple terms the gene amplification model of *CUP1* suggests that, due to the proposed homology of DNA sequences at the junction of *CUP1* repeats, one or multiple units are looped out (Fig. 2). If the loop is replicated, the copy number increases (amplification). If the loop is degraded, the copy number decreases (deamplification).

Fogel and co-workers (22) analyzed 129 tetrads and subjected the DNA to hybridization analysis to determine the changes in copy number of the *CUP1* locus. The results indicate that *CUP1* gene amplification takes place via nonreciprocal recombination or gene conversion. Fogel has proposed a model in which a single strand from one of the *CUP1* repeats invokes a misaligned synaptic pairing with the other chromatid repeat such that all of the information is transferred between synaptic junctions to the unique flanking sequence of the amplified locus in the form of a loop (Fig. 2) (22). Subsequent repair replication of the loop (single strand) leads to an increase in the *CUP1* element depending

upon the number of repeats present in the loop. If no repair/replication occurs, the loop may be excised, and there is a loss of *CUP1* copy number or deamplification. The validity of this model is strengthened by the observation that, in crosses between multiple-copy *CUP1* and single-copy strains, the loop formation can decrease the number of iterations in a multiple-copy strand but cannot increase it. If it is assumed that heteroduplexes form only between nonsister chromatids, then the loop must be stabilized by base pairing in the unique region and base pairing in the reiterated sequences on the other side. Thus, the sequence in the single-copy strain is base paired and cannot form a loop. This model is also consistent with the observation that the frequency of amplification of a single *CUP1* locus in diploid or haploid cells is very low due to the inability of the single repeat to form loops with the other chromatid.

In a majority of the cases, amplification of the *CUP1* locus involves entire repeat units. No deletion or insertion of any DNA has been observed in a *CUP1* repeat unit. Conversional mechanisms that generate copy number changes are known to account for deletion or insertion of DNA. However, in the case of *CUP1* amplification, the conversional events take place at high fidelity and errors in heteroduplex formation and resolution are extremely low.

The model for the mechanisms of *CUP1* amplification described, discussed above and shown in Fig. 2, has been proposed from data derived from crosses of different yeast strains in the absence of copper. It is probable that the principle of amplification remains the same in the presence of copper. In mammalian cells, amplification of the dihydrofolate reductase gene in a single S-phase of the cell cycle (80) leads to the suggestion that disproportionate replication of the locus occurs, followed by a rearrangement/recombination of the amplified DNA to generate elevated levels of the gene and the gene product. The amplified portion of the dihydrofolate reductase DNA can be excised from the chromosomes and can exist as independent units known as double-minute chromosomes (80). No evidence of similar DNA elements containing the *CUP1* locus has been presented in yeasts yet. For disproportionate replication to be applicable as one of the mechanisms of *CUP1* amplification, the locus may either have a sequence which acts as origin of replication or undergo replicon misfiring under the selection pressure of copper.

Future studies of amplification mechanisms would have to address two issues. Does amplification of the *CUP1* locus under high copper concentration follow the pattern as described by Fogel and co-workers (22)? In mammalian cells, the amplification is increased severalfold by addition of DNA-reactive agents or other drugs. What effect do these drugs have on the mechanism of gene amplification in yeast *CUP1*? It is probable that additional mechanisms, analogous to the mammalian system, will be identified in yeasts. The *CUP1* locus contains another gene which codes for a protein X of unknown function in addition to yeast MT. What role, if any, is played by protein X? Since *CUP1* repeats smaller than 2.00 kb have been identified in wine-making yeast cells (22), which presumably do not contain protein X, it appears that protein X gene may not have any role in amplification of the *CUP1* locus. The problem of *CUP1* amplification is well poised for molecular analysis, especially when the repeat unit and the unique flanking DNA sequences have been cloned and sequenced. Cloning and sequencing of additional *CUP1* loci which are related to the standard laboratory *S. cerevisiae* *CUP1* will reveal new information about DNA sequences involved in amplification mechanisms. In addi-

tion, crossing of yeasts with dissimilar *CUP1* (structurally as well as in number of *CUP1* repeats) and subsequent tetrad analysis would also be a guide to the mechanism of *CUP1* amplification.

FUNGAL MTs

Properties of *S. cerevisiae* MT

The MT molecules from *S. cerevisiae* were first described in 1975 (70, 71). The chemical characteristics of the protein, low molecular weight and rich in cysteine, qualified it to be designated copper-thionein (71) or copper chelatin (70). Although the structure of MT was elucidated by DNA sequence analysis of the gene, the detailed structure of the protein molecule is not as well advanced as that of mammalian proteins. Mammalian MT has been the subject of intense biophysical and biochemical studies. The structure of mammalian MT has been resolved in solution by ^{113}Cd -nuclear magnetic resonance analysis (3), and, more recently, the crystal structure of rat liver Cd-Zn MT has been resolved at the 0.23-nm level (25).

Detailed biochemical and structural properties of yeast MT have been reported recently (2, 34, 93, 94). The purified protein was subjected to amino acid sequence analysis and found to lack the first nine amino acid residues whose presence was predicted from the DNA sequence analysis of the gene. This is quite an interesting finding as thus far none of the mammalian MTs have been known to be processed after translation. Also, the only two aromatic amino acids (phenylalanines) predicted from the DNA sequence are present in the first nine residues, which are cleaved in the post-translation processing of the molecule. The functional significance of the leader peptide is not clear as yet. Since five of nine residues of the processed peptide are hydrophobic, it has been suggested that the leader may play a role in compartmentalization of the protein (94). Removal of the first nine residues yields the mature yeast MT, a 53-residue polypeptide of molecular weight 5,655 with glutamine as amino terminus (94).

The metal composition of MT is in part determined by the extent of exposure of the organism to different metals. Since, in the case of *S. cerevisiae*, the MT gene is inducible only by copper, the purified MT yields a protein rich in copper. Up to 60% of the cellular copper can be recovered as copper MT. The isolated protein contains 8 mol of copper ligated to 12 cysteines per mol of protein (94). The demetalated protein is extremely sensitive to the attack of proteases, suggesting the lack of tertiary structure. Reconstitution of copper to the protein suggested that copper exists in the state of Cu(I). These findings are in agreement with original observation that copper exists in the protein as thiolate clusters where the neighboring metal ions are linked with one or more sulfur (93). In addition to copper, the yeast MT also binds Ag(I), Cd(II), Zn(II), and Co(II) (16, 34, 94). The stoichiometry of Ag(I) binding is similar to that of Cu(I). Cd(II) and Zn(II) also coordinate with yeast MT with maximal stoichiometry of four metal ions per mole of protein.

Mammalian MT coordinates 7 Zn(II) or 7 Cd(II) or 12 Cu(I); however, the yeast protein coordinates 4 Zn(I) and 8 Cu(I) ions. The crystal structure studies as well as ^{113}Cd -nuclear magnetic resonance studies have proven that the mammalian protein exists as a two-domain molecule. The β domain extends up to 29 residues from the amino terminus and coordinates 3 Cd ions per 9 cysteine ions. The α domain spans from residue 31 to the carboxy terminus of the protein,

coordinating 4 Cd ions per 11 cysteine ions (3, 25). The evidence for separate metal cysteine thiolate clusters has been strengthened by the fact that subtilin, a protease, cleaves the intact MT into α and β domains with equal amounts of metal bound as described above (68). It has been shown that Cd and Zn are tetrahedrally coordinated to 4-cysteine thiolate complexes in mammalian MT, with 8 of the 20 ligating cysteines probably existing as bridging sulfur in the cluster (3, 40). The higher binding stoichiometry of Cu(I) to mammalian MT strongly suggests that the conformation of the protein is different from Cd-Zn MT. Similarly, reconstitution of yeast MT with Ag(I), Cd(II), and Cu(I) followed by ultraviolet absorption spectroscopy reveals an increase in absorption as a function of added metal. Maximal absorption is observed when 4 mol of Cd(II) bond per mol of protein and 7 to 8 mol of Ag(I) and Cu(I) bond per mol of protein (94). The ability of several metals to protect MT from proteolytic cleavage is another tool to test the metal coordination state of the protein. Maximal protection is rendered when saturating Cu(I), Ag(I), Cd(II), and Zn(II) are coordinated with yeast MT as described above (94). These studies unequivocally demonstrate that, even though the yeast MT gene activation has specific requirements for copper, once the protein is produced, it will bind most of the transition metals. Ultraviolet spectroscopy reveals that binding of different metals generates a molecule of different conformation (94).

Does yeast MT bind copper in two distinct domains as is the case with mammalian MTs? The answer to this question and to the precise geometry of copper in yeast MT requires solution nuclear magnetic resonance studies or X-ray crystal structure analysis. A casual view of the structure of yeast MT indicates that, like *Neurospora* MT, it may possess a single domain. Linear alignment with computer programs fails to reveal any significant homology of yeast MT to mammalian MTs. The only stretch of amino acid sequence which shares homology is Lys-Lys-Ser-Cys-Cys-Ser, present in the center of mammalian MTs (Fig. 3). It is noteworthy that the above sequence acts as a breakpoint which separates the α and β domains of mammalian MTs; however, in yeast MT, this sequence is present three residues before the end of the carboxy terminus of the protein. It appears, therefore, since naturally occurring yeast MT is found as a copper protein, that eight molecules of copper may be located in a single domain of the protein. It is also noteworthy that the amino acid sequence Lys-Lys-Ser-Cys-Cys-Ser, conserved in mammalian and yeast MT, is absent in sea urchin, *Drosophila*, *Neurospora*, and crab MTs (67).

Copper-MT from *N. crassa*

The 26-amino acid residue, a cysteine-rich copper-binding protein, has been purified from the ascomycete *N. crassa* (56). The protein contains only seven different amino acids, and 28% of the protein is composed of cysteine (56). The organization of cysteine residue in *N. crassa* protein is exactly the same as the amino-terminal region of mammalian proteins. Data based upon 250-nm absorption and electron paramagnetic resonance spectroscopy of the freshly isolated copper loaded protein suggest that copper is present as a cuprous form, and all of the cysteines are ligated to form Cu(I) complexes with six copper molecules per mole of protein (56). The *Neurospora* protein has not been crystallized, and no detailed information is available on the protein structure.

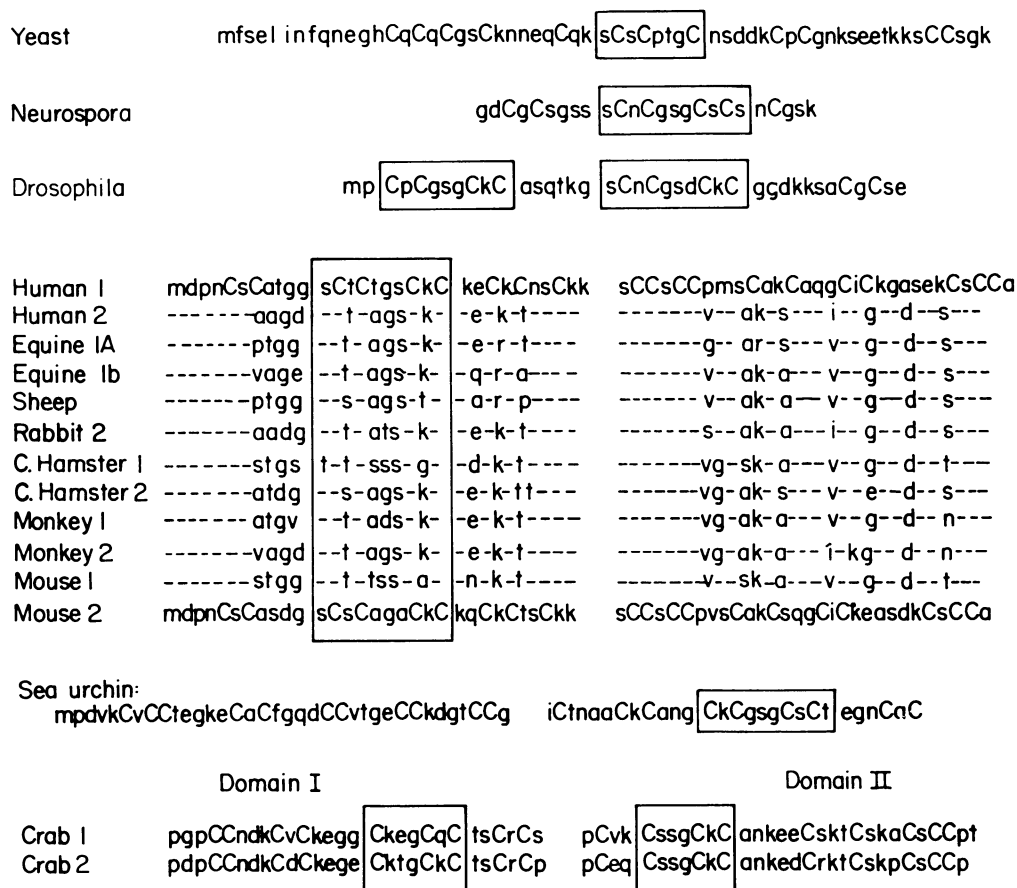


FIG. 3. Conservation of the central segment, a model for evolution of the MT gene. Other MT sequences are human MT1 (76), human MT2 (49), equine MT1A (50), equine MT1B (49), mouse MT1 (26), mouse MT2 (39), sheep MT1 (69), rabbit MT2 (43), Chinese hamster MT1 and MT2 (28), monkey MT1 and MT2 (81), *Neurospora* MT (56, 62), yeast MT (8, 46), crab MT1 and MT2 (57), *Drosophila* MT (55), and sea urchin MT (67). The central segment, described in the text, is enclosed in a box. One-letter code: a, alanine; C, cysteine; d, aspartic acid; e, glutamic acid; f, phenylalanine; g, glycine; h, histidine; i, isoleucine; k, lysine; l, leucine; m, methionine; n, asparagine; p, proline; q, glutamine; r, arginine; s, serine; t, threonine; v, valine (note that only cysteine is represented by an uppercase letter for emphasis). (Adapted from reference 67.)

Cadmium-Inducible Proteins from *Schizosaccharomyces pombe* and Plants

Thus far, there are no reports for copper-inducible cysteine-rich protein isolated from *Schizosaccharomyces pombe* (see below). Search for metal-binding polypeptides has led to the discovery of cadmium-binding proteins from *Schizosaccharomyces pombe* (52, 63). Murasugi et al. (64) show that prolonged treatment of *Schizosaccharomyces pombe* with 0.5 mM CdCl₂ induces two kinds of Cd-binding peptide (Cd-BP1 and Cd-BP2) (64). Initial studies show that the Cd-BP is composed of a unit peptide which Kondo et al. (52) have named Cadystin. Earlier studies indicate the following structure for the unit peptide (52): H-γ-Glu-Cys-γ-Glu-Cys-Cys-γ-Glu-Gly-OH. However, more recent studies by Grill et al. (30) and revisions of the previously proposed structure (51) have evolved a new structure and a proposed name of "phytochelatin" for the sulfur-rich heavy-metal-binding polypeptides (30). In agreement with the previous studies, the metal-binding peptides are composed of only three amino acids, namely, L cysteine, L-glutamic acid, and glycine. Glutamic acid is linked to each cysteine by a γ-peptide linkage. Therefore, phytochelatin cannot be regarded as primary gene products.

Similar peptides have also been observed when plant cells are treated with heavy metals such as Cd, Cu, Hg, Pb, and Zn (29). The general structure of these peptides is (γ-Glu-Cys)_nGly (*n* = 2 to 7). It has been suggested that phytochelatin are present in all plant species belonging to *Angiospermae* and *Gymnospermae* (29). No phytochelatin-like metal-binding peptides have been observed in *S. cerevisiae*. However, the related yeast *Schizosaccharomyces pombe* contains two peptides which have a structure surprisingly similar to those of the plant phytochelatin (γ-Glu-Cys)_nGly (*n* = 2 and 3) when the *Schizosaccharomyces pombe* cells are treated with Cd (30). It appears that the two peptides identified in *Schizosaccharomyces pombe* by Grill et al. (30) are the same as Cd-BP1 and Cd-BP2 previously characterized by Murasugi et al. (64).

In addition to predominant forms of Cadystin A and B, corresponding to phytochelatin *n* = 2 and 3, five additional homologous peptides with chain lengths ranging from *n* = 4 to 8 are also observed in *Schizosaccharomyces pombe*. The kinetics of induction of different phytochelatin in plants and yeasts support the suggestion that these peptides are synthesized by elongation of the peptide with one (γ-Glu-Cys) unit at the expense of and possibly starting from glutathione (29, 30).

Thus far, there are no reports for the presence of a Cd- or Cu-inducible MT molecule coded by a gene in *Schizosaccharomyces pombe*. In this respect, *S. pombe* is more closely related to plants which do not induce MT. However, these cysteine-rich peptides do bear some resemblance to MT as they can sequester several transition metals, and thus they constitute a new class of natural compounds.

Unlike the induction of MT genes in the mammalian system and *S. cerevisiae*, the metal detoxification process in plants and *Schizosaccharomyces pombe* is accomplished by the synthesis of metal-sequestering peptides. The copper resistance in *S. cerevisiae* is efficiently regulated by the induction of a single MT gene. It appears that the metal homeostasis system evolved in plants and *Schizosaccharomyces pombe* shares some common features with MTs. Multiple enzymes may be involved in the biosynthesis of phytochelatins, and mechanisms must exist for metal-dependent induction or activation of the enzymes involved in biosynthesis of phytochelatins.

Copper-Inducible MT from Human Pathogenic Fungi

Opportunist human fungal infections are a major problem in the clinic. Human pathogenic fungi do not exist as a haploid in different stages of their life cycle. Consequently, the genetic study of these fungi is not as well advanced as that of *S. cerevisiae*. Six different clinical isolates of the pathogenic fungi *C. albicans* and *C. glabrata* have been analyzed for the induction of MT-like protein in response to copper, cadmium, zinc, and gold (10). None of the pathogenic fungi produced a low-molecular-weight cysteine-rich protein in response to cadmium, zinc, or gold. *C. albicans* strain 792 produced a protein similar to that found in *S. cerevisiae* in response to copper, and in *C. glabrata* strain 62 a unique, approximately 4,500-dalton protein was induced by copper. The presence of a copper-inducible locus in *C. glabrata* 62 might suggest that it has evolved from *S. cerevisiae*; however, lack of DNA sequence homology with the Cu-MT gene of *S. cerevisiae* suggests otherwise. Detailed analysis of relationship between different pathogenic fungi will have to wait for the cloning of an MT-like gene from pathogenic fungi. The cloned MT-like gene will be useful as a selectable marker and for regulated gene expression studies in pathogenic fungi.

Evolution of MTs

Are MTs of higher eucaryotes products of convergent or divergent evolution? What are the determining forces which have preserved the primary sequence of the protein? Since the important amino acid is cysteine, an arrangement of cysteine residues in a way which ligates or bridges the ligated metal would fulfill the function of the protein. This hypothesis is consistent with the notion that a single MT can ligate different metals and attain different conformations. However, if MT molecules play a more complex function in the cell (i.e., transfer of specific metal to metalloenzymes), then a distinct tertiary structure is required which would allow the molecule to participate in a metal-specific function. A tertiary structure, in turn, requires that at least the arrangement of cysteine be conserved, and such a conservation has been postulated (67). It is proposed that the key to the evolution of the family of MT proteins is the relatively unchanged central segment (67). The central segments of different MTs have the following characteristics: (i) on one side are the four residues X-Cys-X-Cys or Cys-X-Cys-X,

where X = Ser or Thr; (ii) Cys-Lys-Cys is on the other side; and (iii) a triplet of noncysteine amino acids is in the middle. A similar segment is found in *Neurospora* MT and two such segments, with only conservative substitutions, are found in *Drosophila* MT. Furthermore, the middle triplet is the Gly-Ser-Gly for the segments from sea urchin, *Neurospora* spp., and *Drosophila* spp., and although this middle triplet is highly variable in the mammalian segment, it is restricted to combinations of alanine, threonine, glycine, and serine. In the MTs of two other species, the segment appears to be only partially conserved; in yeast MT, it lacks the Lys-Cys in the Cys-Lys-Cys group, whereas in crab MT it lacks the X-Cys in the X-Cys-X-Cys group. The designation "central segment" can be seen as appropriate because it is located near the center of yeast and *Neurospora* MTs, in the center of the mammalian β domain, and in the center of the counterpart sea urchin carboxyl-terminal half of MT. Since the central segment is present in the center of yeast MT and because of the lack of cysteine residue arrangement as seen in the α domain of mammalian MTs, it appears that the yeast MT may contain a single metal-binding domain. In the case of *Drosophila* MT, in which the central segment occurs twice, one of these segments is, indeed, positioned centrally. The segment occurs off-center in each of the two domains of crab MT.

The central segment of *Neurospora* MT can be represented as an 11-amino acid sequence (Ser-Cys-Asn-Cys-Gly-Ser-Gly-Cys-Ser-Cys-Ser) that is symmetrical (Fig. 3), except for a difference in one residue, and, considering the primitivity of the organism, might be a prototype of an evolutionary progenitor segment. The *Neurospora* MT amino-terminal portion is conserved in *Drosophila* and, with only one substitution, in yeasts and mammals. The carboxy-terminal portion of *Neurospora* MT is conserved in the sea urchin. Single amino acid replacements in a progenitor segment can give rise to distinctive central segments. The reversed polarity (the side on which the Cys-Lys-Cys group resides relative to the middle triplet) of sea urchin central segment could be the result of a single substitution, lysine replacing the asparagine of *Neurospora* MT. Replacement of serine by lysine converts the *Neurospora* central segment to that of *Drosophila* spp. The latter segment is only one replacement step away from a characteristically mammalian central segment. The polarity of the central segment in *Drosophila*, crab, and mammalian MT molecules is the same as in *Neurospora* MT.

Although it is difficult to draw any evolutionary relationships between the MTs discussed, it might be useful to propose that the central segment be considered the core upon which a diversity of MTs has been based. The high degree of conservation of both the location and amino acid sequence in MTs from many sources argues for evolutionary importance of the central segment.

COPPER-INDUCIBLE TRANSCRIPTION SYSTEM FROM *S. CEREVISIAE*

The discovery and much of the original work on MTs is owed to biophysicists, who are interested in the chemistry of metals and protein interaction, and to the pharmacologists, who are interested in metal metabolism. More recently, much attention has centered around MT as a model for studying mechanisms of gene regulation. Cloning of fungal, as well as mammalian, MT genes and identification of regulatory signals have made these genes an important tool for the genetic engineer. This section deals with our current

understanding of the mechanisms of yeast MT gene expression.

UAS of the *S. cerevisiae* MT Gene

In eucaryotes, progress in understanding regulation of transcription has come from identification of *cis*-acting DNA sequences which are adjacent to the transcription initiation site. The yeast MT gene resembles other eucaryotic genes transcribed by RNA polymerase II (31, 60) in which, 20 to 100 base pairs (bp) upstream of the initiation sites, TATA-like sequences determine the initiation of transcription. Analysis of the metal-regulated transcription of yeast MT has been facilitated by fusing the upstream activating sequences (UAS) of the gene with the *Escherichia coli* galactokinase gene (*galK*). This gene fusion was inserted in a yeast vector which contains the yeast *TRP1* gene as a selectable marker and *ARS1* and *CEN3* sequences to promote stable low-copy replication in cells (8). Transformation of this plasmid into a yeast strain allows a copper-mediated increase in *galK* activity. A 430-bp fragment of MT 5'-flanking sequence (from the ATG) was identified to contain all of the information required for transcriptional activation and copper induction (8).

A common approach to identify the *cis*-acting DNA sequences involves progressive deletion from the 3' or 5' end of the UAS. The deletion mutants are fused to the *E. coli galK* and the regulation of expression is analyzed as above. Such a deletion analysis has established that the sequences between -105 and -180 bp from the transcription initiation site of the yeast MT gene are essential for efficient copper-mediated expression of *galK* (89). These studies also conclude that there are at least two functional elements residing in this control region. A third sequence which is under the negative control (activation by derepression) may reside between -100 bp and the transcription initiation site (89). It is very likely that multiple copies of the metal-responsive DNA sequences are present on the yeast MT UAS. In fact, the presence of multiple upstream control elements is a common feature of many yeast genes (31, 37) and higher eucaryotes including MT (87, 88). Analysis of the yeast MT 5'-flanking sequence led to the identification of 32- and 34-bp sequences which are repeated twice (89). To test that the metal-regulated DNA element resides in these sequences, one of the repeats was synthesized. When a synthetic repeat is inserted into the *galK* expression vector, it results in a 10-fold increase of basal expression level and a 1.8-fold increase following addition of copper. The same repeat sequence, when inserted in the opposite orientation (unnatural), results in a 2-fold increase by copper; however, the basal expression is 50% of that observed in correct orientation. When two copies of the synthetic repeat sequence are inserted in tandem in the expression vector, the result is a 40-fold activation of basal transcription with 3.3-fold induction ratio with added copper (89). Insertion of the repeat sequences in opposite orientation leads to a 24-fold increase in activity; however, no further increase is observed after copper treatment. Independent of the above functional studies, a computer-aided analysis of the 5'-flanking DNA sequence of the yeast MT gene reveals six repeats of 5' TCTTTTGTCT 3' (the consensus sequence). Some of the elements are present as direct repeats, while a few are inverted repeats (D. Ecker, unpublished observations). This consensus sequence is also present in the synthetic element tested for copper-mediated regulation of transcription (89).

We note that the synthetic elements tested contain two inverted repeats of the consensus sequence (89). It is also

noteworthy that the consensus sequence of mammalian MT, 5' CTNTGCRCNCGGCCC 3', is radically different from that of yeast MT gene (88). However, like the mammalian MT consensus sequence, the yeast consensus sequence functions maximally when placed as a tandem inverted repeat unit (82, 89). Thus, it appears that the consensus sequence of yeast and mammalian MT are distinct from each other. However, the arrangement of the consensus sequence in the regulatory sequences is remarkably similar. It can be said with a reasonable certainty that the MT regulatory sequences will not be functional in a heterologous system. It can be speculated further that the *trans*-acting regulatory elements of the MT system are also distinct in higher eucaryotes and yeasts.

Regulation of MT Gene Expression

In the mammalian system, MT genes are subject to regulation by multiple stimuli, including glucocorticoid hormones, lymphokines, stress, and a variety of transition metals (32). The copper MT gene of *S. cerevisiae* has obvious advantages for classical and modern genetic studies because the yeast MT is regulated by only a single metal ion, copper (8, 16). Since a multicopy MT-*galK* fusion vector is appropriately regulated in CUP1⁺ yeast strains, it has been proposed that the *trans*-acting regulatory factors are not rate limiting (27). The first clue for the role of a *trans*-acting factor was obtained when yeast cells lacking the intact chromosomal MT locus (*cup1Δ*) expressed high levels of *galK* from the MT-*galK* expression vector even in the absence of copper (27, 33). These results are explained on the basis of a general model that the metal-free protein apothionein, a negative regulator of the gene, represses the transcription by binding to the promoter sequence of the gene (autoregulation) (47). In this model, the transcription is proposed to be initiated by metal binding to the thionein to form MT, an inactive repressor. Initially, this model for regulation was thought to be reasonably simple and economical for the cell as the levels of apothionein and MT regulated their own gene expression. However, the yeast protein is capable of binding metals other than copper, and yet the gene is induced only by copper (8, 16, 34, 93, 94).

Several lines of evidence suggest that, at least in *S. cerevisiae*, MT gene transcription may be regulated in a positive manner by a copper-specific *trans*-acting factor(s). First, expression of monkey MT under the control of yeast MT UAS in a *cup1Δ* yeast strain is appropriately induced by copper and not by any other metal ion (16, 90). It appears that monkey MT, which is structurally fairly divergent from yeast MT (except for a small stretch of amino acid sequence [8]), is able to repress yeast MT gene activation. Second, the yeast MT binds copper, cadmium, zinc, silver, and cobalt (16, 34, 95). Of these metals, only copper induces transcription of the gene (16). Of all the above-mentioned metals, only cadmium is transported in the cell and binds yeast MT, and yet it is unable to induce the gene transcription (16). These observations, coupled with the fact that pure yeast MT does not bind to its promoter sequences (90), strongly suggest that the MT does not directly regulate its gene expression.

Recent studies indicate that the yeast MT gene may also be under a negative control (P. Grossfeld and R. Butow, unpublished results). Deletion of 5'-end sequences of the MT gene beyond -100 bp (upstream) completely abolished the copper-mediated induction of the MT gene in the presence of glucose as a carbon source. However, when the yeast strains containing the deleted MT plasmids were transferred from

glucose as a sole carbon source to raffinose or galactose, a fivefold increase in resistance towards cadmium was observed. Cadmium does not regulate the gene; however, MT binds cadmium and confers resistance. The use of cadmium is very important for these studies as it separates gene regulation from the gene function, which is not possible with copper. These results suggest the presence of glucose-repressible DNA sequences between initiation of transcription and -100 bp of the MT gene. In this respect, the MT gene may be similar to the *CYC1* gene which is also glucose repressed (31). The suggestion that the MT gene may also be under a negative control is supported by the observation that deletion of sequences downstream from the -100 bp resulted in increased basal expression (89). These studies were performed in the presence of glucose, and thus it is likely that a glucose-repressible sequence is present in the MT gene. These studies, like those with mammalian MT, suggest that the yeast MT gene transcription is also under the control of multiple stimuli. However, negative control of the MT gene as a mechanism of gene activation has not been reported yet for the mammalian MT genes.

A model for MT gene regulation has been proposed which states that autoregulation of yeast MT synthesis is the result of the copper-binding properties of the protein which lower the free intracellular copper concentration, thereby dissociating the copper and copper-specific *trans*-acting transcription factor (Fig. 4). In this model, the inactive *trans*-acting factor (round shape) is activated (square shape) after copper binding which restores the transcriptional activity. The model also states that a repressor site is present in between -100 bp and initiation of the transcription site of the MT gene. Dissociation of a repressor is promoted by the removal of glucose from the media. MT gene transcription is derepressed in the presence of galactose as a sole carbon source (see above). However, the MT gene can be induced by copper, even in the presence of glucose. The copper-mediated activation of transcription presumably overrides the glucose-mediated repression of the gene.

The possibility that free copper may bind directly to the promoter sequences to regulate the transcription cannot be excluded. In the model described in Fig. 4, the transcription activation circuit is broken when the level of free copper is lowered by MT sequestration. It is likely that the *trans*-acting factor recognition sequence is the consensus sequence repeated at least five times in the UAS of yeast MT (between -54 and -400 bp). At least two of the consensus sequences of the UAS are present on the synthetic sequence and are responsible for copper-mediated transcription of the *galK* sequence (89). That the transcription is induced in a positive fashion is evident from deletion analysis of the UAS which delineated two positive activating sites (89). Insertion of a single consensus sequence in the *galK* expression vector did not stimulate transcription in the absence or presence of copper, indicating that, as in mammalian systems, in yeasts the consensus sequence is operational when present in tandem and as a direct repeat (87, 89; D. J. Ecker and L. W. Bergman, unpublished results).

As discussed above, the lack of MT induction by cadmium in yeasts points to the existence of one or more *trans*-acting factors which are specific for copper. It also indicates that MT protein acts as an indirect autoregulator of its own gene expression by controlling the free cellular copper. Thus, increasing the number of chromosomal *CUP1* genes should restore greater repression of episomal MT-regulated gene transcription. This hypothesis has been confirmed by examining the *galK* level in a *cup1*^Δ strain which carries MT UAS

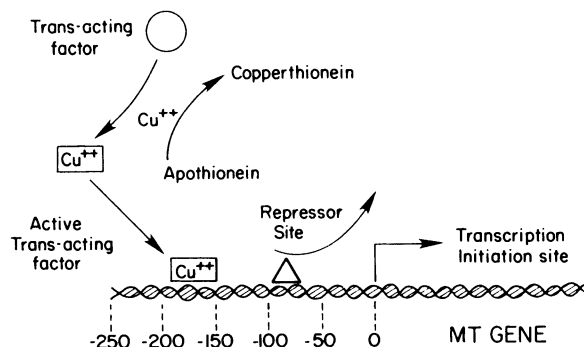


FIG. 4. Model for *trans*-acting copper-mediated regulation of MT gene transcription.

fused to *galK*. When this strain was cotransformed with a vector in which yeast MT was constitutively expressed under the control of the yeast glyceraldehyde-3-phosphate dehydrogenase (TDH) promoter, the *galK* activity was dramatically reduced (27).

Current research is directed toward identification of the protein factor which stimulates copper-specific transcription. It is likely that transcription of yeast MT is regulated by the interaction of multiple components with the UAS. We can envisage protein factors interacting with the transcriptional complex after the change in conformation which results from binding copper. Binding of other metals does not afford a desired geometry, and the metals act as an antagonist and further depress the basal level of transcription. Inhibition of the basal level of MT transcription by cadmium is consistent with this model (16; D. Ecker and T. R. Butt, unpublished results). Crystallization of the *trans*-acting factor with copper will lead to detailed molecular structure and mechanisms of metal-mediated regulation of transcription. It is the wish of the genetic engineer and the molecular biologist to design regulatory proteins which can be regulated by metals other than copper.

COMMERCIAL APPLICATIONS OF THE *CUP1* LOCUS IN BIOTECHNOLOGY

Yeasts have been the most important commercially exploitable microorganisms since ancient times. Because fermentation technology for yeasts is well established, and they are safely consumed by mankind, the value of yeasts in biopharmaceutical and other biotechnology fields has been greatly enhanced.

Importance of the *CUP1* Locus in Heterologous Gene Expression

The genes for glycolytic pathway enzymes are constitutively expressed for the continuous metabolism of glucose and fructose. Because the glycolytic pathway enzymes represent up to 65% of the total cellular protein (24, 36), these genes were among the first few cloned by recombinant DNA techniques. It had been assumed that the promoter for the glycolytic enzyme genes would be efficient in expressing heterologous gene products. Experience from heterologous gene expression studies in *E. coli* shows that the efficient expression of a protein product is governed by several factors including strength of the promoter, stability of the messenger RNA, nature and structure of context effect (the

junction between promoter sequences and the structural gene), the host strain used, lethality of the protein to the host, stability of the protein product, and a number of other factors (78). Early studies indicate that the promoter sequences from genes of the yeast glycolytic pathway enzymes TDH and phosphoglycerate kinase (PGK) are among the most efficient promoters of yeasts (11, 91). Further work shows that use of an efficient yeast promoter does not necessarily guarantee an increase in expression of the heterologous gene product. Thus, while the PGK enzyme may account for 18% of the total cellular protein under the homologous promoter, the expression of human interferon is no more than 0.5% of the total cellular proteins (11). To date, quite a few yeast expression systems have been described which allow regulated expression of the heterologous genes. The *PHO5* gene encodes for an alkaline phosphatase which is inducible upon growth of cells in phosphate-depleted media (53). Similarly, the *GAL1* gene is induced when the yeasts are grown in a medium containing galactose (1). However, the use of these promoter systems has not yielded efficient expression of heterologous products (53), and the rate of transcription is not comparable to that of the constitutively expressed glycolytic pathway promoters.

The MT promoter from yeast *CUP1* satisfies many of the criteria for an efficient and regulated yeast promoter. The discovery of the copper-regulated MT promoter is quite recent, and considerable work is needed to understand the precise mechanisms of gene regulation. The progress and recent results on the *CUP1* expression system indicate that it may be one of the best expression systems discovered in *S. cerevisiae*. Much of the work on the MT promoter from *CUP1* in yeasts is of a proprietary nature and yet to be published; however, a general picture emerges which indicates that the promoter system has the following advantages.

(i) The MT UAS is under the control of copper and the addition of copper to the media induces a transcription of the gene up to 2⁵-fold higher than the basal level (8, 16, 27). Our understanding of the mechanism of gene induction indicates that the level of free intracellular copper determines the fold increase observed with respect to the basal expression level. The best induction ratios are observed in synthetic medium which is low in endogenous copper concentration. No published data are available on induction efficiency of MT UAS when cells are grown in rich media; however, the principle of gene activation in response to free intracellular copper should also apply in this case. Due to several copper-binding components present in rich media, the copper concentration required for maximal induction is expected to be in the millimolar range. If the yeast MT gene is glucose repressed as indicated by the preliminary data (see preceding section), then the growth of yeast cells in the presence of copper and a carbon source other than glucose may further increase the level of transcription.

Low levels of copper are nontoxic for human consumption. Use of copper as an inducer of heterologous gene product in *S. cerevisiae* should not present a hazard for the regulatory agencies for the approval of biopharmaceuticals produced in yeasts. As compared to the inducible systems of galactose or phosphatase genes, copper is of low molecular weight and is an extremely inexpensive inducer; it thus proves to be economical.

(ii) In some instances, the product of a foreign gene happens to be lethal for the host. Under these circumstances, it is desirable to construct the recombinant strain in which the particular gene expression is repressed. Following appropriate growth and scale up, the promoter system is

induced with copper for an allowed period of time in the fermentor and the product is recovered thereafter.

Several wild-type yeast strains contain multiple copies of the *CUP1* locus. As discussed in the previous section, MT represses its gene transcription, and the induction ratio of the plasmid-borne MT gene fusion expression is directly proportional to the number of MT genes present on the yeast chromosomes (16, 27). When a *cup1* Δ strain is used as host, the MT UAS-mediated transcription is partially constitutive in the absence of copper (16, 27). Addition of copper further stimulates transcription of the MT UAS-fused gene (27). The basal level of transcription is further repressed when a *CUP1*⁺ yeast strain is used for heterologous expression, and a complete repression to basal level is observed when a chromosomally amplified *CUP1*⁺ strain is used (27, 33). Thus, the *CUP1* gene expression in the yeast system offers a variety of basal and inducible transcription activities which can be conveniently selected by choosing an appropriate yeast strain.

(iii) The strength or efficiency of a promoter is defined as the number of times RNA polymerase can transcribe the gene during a period of time. In this respect, the yeast TDH and PGK promoters are considered quite efficient. A direct comparison of yeast TDH and MT promoters has been made by fusing the UAS with the *E. coli galK* gene and assaying *galK* activity as a measure of promoter strength. Results from single-copy as well as multicopy plasmids in a *CUP1*⁺ background, after copper addition, show that the MT promoter is equally or 20% more efficient than the TDH promoter (77). In a different set of experiments, MT promoter strength is compared with the PGK gene on multicopy plasmids in which the UAS is fused to a human serum albumin gene (18; T. Etcheverry, W. C. Forrester, and R. A. Hitzman, unpublished data). The results show that under fully inducible conditions the MT promoter mediates the synthesis of human serum albumin, which is 18% of the total yeast protein (Etcheverry et al., unpublished data). This is equal to the amount of the protein synthesized under the control of the PGK promoter (Etcheverry et al., unpublished data).

These results demonstrate that under fully inducible conditions the MT promoter is equal to, or more efficient than, two of the glycolytic promoters, TDH and PGK. Another unique property of the MT promoter is that, in addition to being highly efficient, the promoter is also inducible while the glycolytic promoters are constitutive. To date several proteins have been expressed in the *CUP1* expression system for commercial and research purposes. They include human serum albumin (18), human ubiquitin gene (17), *E. coli galK* gene (8), monkey MT-I and MT-II (16, 90), mutant yeast MT (16), and human hepatitis virus antigen (unpublished results).

Applications of Yeast MT System in Research

The differential gradation of the copper resistance observed is directly controlled by the copy number of the MT gene or the efficiency of MT expression. For this reason, yeast MT is an invaluable tool in yeast genetics. This unique property of the yeast MT has been exploited to analyze the role of centromere sequences in chromosome 3 (CEN3). Circular yeast plasmid (which contain the MT structural gene in the following arrangement: *GAL1* promoter . . . CEN3 . . . MT . . . *ARS1* . . . *TRP1*) were transformed to a *cup1* Δ strain and single colonies were selected from growth on media with increasing copper concentrations (75). The

authors conclude that the variability in copper resistance is related to the copy number of CEN3 (up to 10 per cell), of which there is normally one copy per cell. Thus, the MT offers an opportunity to analyze and isolate the structure(s) involved in the function of CEN3 by isolating mutants and selecting with MT as a reporter gene. A similar approach has been used to construct promoter libraries from yeasts in which the structural MT gene is used as an indicator. This approach offers an opportunity to identify and isolate DNA sequences which are regulated under different genetic backgrounds or various stimuli. The strategy has been successfully applied to isolated nuclear DNA sequences which are regulated by yeast mitochondria (R. Butow, unpublished results). MT fused to mitochondrial F₁ adenosine triphosphatase β subunit has led to the finding that unfolding of the subunit is required for the translocation of mitochondrial proteins (12). Ubiquitin, a highly conserved protein, is covalently conjugated to the target proteins and signals proteolysis of the target proteins by ubiquitin-dependent proteases (19). Fusion of MT to the C terminus of ubiquitin mediates degradation of MT in yeasts. Ubiquitin-MT gene fusion in yeasts provides a powerful genetic system to study mechanisms of selective proteolysis (7). The brewing yeasts are not readily amenable to classical yeast genetics without disturbing the desirable characteristics of the strain. The yeast MT gene has been used successfully to transform several brewing strains (35). Selection with copper is a useful tool to manipulate genetically the commercial brewing strains and improve the characteristics of the product.

CUP1-Mediated Amplification of Foreign Genes on Yeast Chromosomes

It is noteworthy that the repeat structure of the *CUP1* locus is highly conserved in laboratory as well as most commercial strains of *S. cerevisiae* (21, 22, 93). This is because the *CUP1* locus is amplified with a remarkable fidelity (see subsection, "Mechanisms of *CUP1* Gene Amplification"). It is possible to engineer the *CUP1* locus and place the gene of interest at the appropriate site. The linear DNA containing the gene of interest and the uninterrupted MT gene is transferred to the chromosome by homologous recombination, and the yeast strain is selected for growth in increasing concentrations of copper. An amplifiable system should have the following properties, all of which are offered by the *CUP1* locus, making it an excellent vehicle for these studies.

Selection of amplified locus. Since the mechanism of gene amplifications is not completely understood, and it is an infrequent and spontaneous event, the strain containing the amplified resident or foreign gene must be selected. Functional MT genes are required for efficient selection. Because there is a linear relationship between number of MT gene copies in the cell and resistance to copper (21), a yeast strain with 60 tandemly amplified *CUP1* loci can be selected which grows on a synthetic medium containing 5 mM copper sulfate (T. R. Butt, unpublished data).

Tandem amplification. The *CUP1* amplification system also offers the advantage that the unit is amplified at the same site in a tandem fashion. The amplification process appears to take place via gene conversion (22). Faithful replication or repair of the single-strand loop leads to amplification of the exact same unit, and up to 16 kilodaltons of the DNA can be duplicated (58). As discussed previously, the amplification model of Fogel et al. assures that asymmetrical strand transfer to the other chromatid involves

unpaired single-stranded loop formation (22). While loop replication and repair may lead to gene amplification, the loop degradation leads to deamplification (22). In a few cases, deamplification of the *CUP1* locus was observed in unselected tetrads (22). If the mechanisms proposed in this model are assumed to be correct, then in a fraction of cells the loss of the amplified chromosomal unit would present a problem. However, deamplified strains will obviously be selected out by high copper content of the growth medium. The *CUP1* amplification system has been used to amplify stably foreign genes on yeast chromosomes (M. J. Walling and D. H. Hamer, unpublished results).

Importance of Yeast *CUP1* System in Metal Recovery

The value of yeasts in biotechnology is well established, and the importance of the *CUP1* system has been discussed in the preceding section. A potential value of the *CUP1* system and the MT molecule in microbiological mining and metal recovery is also apparent. Considerable information is available on *Thiobacillus ferrooxidans* and another acidophilic bacterium, *Acidiphilium organovorum* (5, 38, 95), which thrive in the acid environment of copper ores. *T. ferrooxidans* can grow on carbon dioxide from air, and the energy released by oxidizing iron and sulfur can be found in metallic sulfides. In a commercial leaching operation, bacterial growth is promoted by spraying water on low-grade ore and recycling the effluent. When the concentration of copper becomes high enough, the metal is removed by electrolysis or precipitation (5, 14). *T. ferrooxidans* leaches metal from ores by oxidation of the metals at the cell surface, using molecular oxygen. This reaction leads to the formation of water and the entrapment of electrons in the cells, where they contribute to the formation of adenosine triphosphate (5). Upon oxidation, the sulfides of metal are more soluble, and the sulfuric acid produced further helps the oxidation of metals. We note that in this mechanism metal does not enter the cell. Although *T. ferrooxidans* has great potential for metal recovery, the biochemistry and genetics of this organism are poorly understood, and only recently have the DNA elements which confer metal resistance been characterized (73, 74, 95).

In the *S. cerevisiae* *CUP1* system, copper is internalized and bound to MT, a pathway radically different from that in *T. ferrooxidans*. The yeast system can be useful if it meets two criteria: the absorption and uptake of the metal and chemical transformation. It would prove difficult to grow *S. cerevisiae* strains in an environment suitable for the growth of *T. ferrooxidans*. Thus, *S. cerevisiae* may not be suitable for ore mining, but it can be useful for metal recovery from a solution under optimum growth conditions.

Due to the current depressed price of copper, recovery of the metal may not be economical at the moment; however, recovery of precious metals such as gold, silver, uranium, and platinum is highly desirable. Two procedures can be devised for the recovery of precious metals; the first depends upon selecting yeast strains which accumulate metal inside the cell. In one of the experiments, the *S. cerevisiae* strain is selected for growth in 10, 50, and 100 μ M gold compounds. Analysis of the cytoplasmic content of the yeast indicates that there is a linear relationship between the gold resistance and uptake of the molecule in the cell (Butt et al., unpublished results). It is known that a gold molecule binds MT in bidentate configuration (54), but gold molecules do not induce yeast MT gene transcription. Yeast strains with constitutive expression of MT genes are expected to accumulate even greater amounts of gold.

The crystal structure of mammalian Cd-Zn MT has been elucidated (25), and yeast Cu-MT crystallization is in progress (Winge et al., unpublished results). The information on the crystal structure has generated enough data to design a new MT (using synthetic DNA) which has appropriate affinity and the maximum ratio of metal to protein molecule. In a deliberately designed MT, when sulfur is not the best coordinating ligand for a metal, nitrogen in the form of histidine can replace the cysteine.

A second approach can be designed for the recovery of precious metals which do not enter the cells. The MT synthesized in cells can be secreted in the periplasmic space or extracellularly by attaching appropriate secretory signal sequences to MT genes (96). To our knowledge, the natural yeast MT is not secreted in the media. Although efficient secretion of MT has not been tested, reports indicate that low-molecular-weight (<10,000) proteins are more efficiently secreted than high-molecular-weight proteins. In several cases, the secreted protein resides in the periplasmic space of yeasts (17). A wide variety of MT molecules can be engineered to have specific affinity for different metals. The yeast loaded with a metal can be centrifuged, the metal can be released by washing, and demetalated cells can be regenerated. In conclusion, the genetics and cell physiology of yeasts is far more advanced than for *T. ferrooxidans* and, coupled with the knowledge of metal-MT interaction, can prove an important tool for the recovery of precious metals.

ACKNOWLEDGMENTS

We thank the following individuals for help in preparation of this review: Seymour Fogel, Ronald Butow, Jerry Gorman, and Paul Grossfeld. Thanks go to Susanne Young for organizing and typing this manuscript.

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